修 士 学 位 論 文

Adaptation of *Lilium auratum* var. *platyphyllum* to nocturnal pollinators in the Izu Islands

伊豆諸島に生育するサクユリ（ユリ科）の夜行性送粉昆虫への適応（英文）

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学位論文要旨（修士（理学））

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昆虫などの動物に送授粉を委ねる動物媒の植物にとって、花は有効な送粉者を誘引するための重要な器官である。伊豆諸島のみに自生するサクユリ（*Lilium auratum* var. *platyphyllum*）は大きな白色の花被片をもち、強くて甘い花香を放出する。また、サクユリは島によって違いがみられ、伊豆諸島北部の島では白い花被片に赤色斑紋のある個体と斑紋のない個体が同所的に生育しているのに対し、南部の島では花被片に斑紋のない白色の花をもつ個体のみが生育している。白色の花被片や甘い花香といった特徴は、一般的に夜行性のガ類を誘引するのに適応した形質と言われている。伊豆諸島に自生するニオイウツギやニオイエビネなどの固有種も強い花香を放出することから、伊豆諸島においては夜行性のガ類が重要な送粉昆虫であると考える研究者もいる。そこで本研究では、花被片に斑紋ありとなしの個体が同所的に生育している島（伊豆大島）と斑紋なし個体のみが生育している島（青ヶ島）の２島のサクユリ、ならびにその基準変種である本州（東京都八王子市、埼玉県嵐山町）産のヤマユリ（*Lilium auratum* var. *auratum*）について、自家和合性の有無や花香強度、蜜に含まれる糖量、訪花昆虫相の日内変化を調べ、サクユリの花形質的特徴と送粉昆虫の関係を明らかにすることを目的とした。

人工授粉実験によって自家和合性の有無を調べた結果、サクユリとヤマユリどちらも強い自家不和合性を示すことが明らかになった。したがって、両変種とも送粉昆虫が繁殖に重要な役割を果たすことがわかった。臭気計によって測定した花香強度については、サクユリの斑紋の有無や島に関わらず、夕方と深夜の２回花香が強くなったのに対して、ヤマユリでは夜のはじめ頃に花香がやや強くなった。どちらも朝には花香は弱くなった。蜜量と糖濃度から算出した蜜に含まれる糖量についても、サクユリでは夕方に最も多くなり、その後しだいに糖量が減っていき昼前に最も少なくなった。一方、ヤマユリでは日内で糖量に大きな変化はみられなかった。デジタルカメラを用いたインターバル撮影

による訪花昆虫の種類については、サクユリでは斑紋の有無での差はみられなかったが、伊豆諸島の２島間ではその種類に違いがみられた。伊豆大島では、夜間に大型のスズメガ類やヤガ類、薄暮期から夜間にかけて小型のスズメガ類が主に訪花していた。青ヶ島では、日中にツマグロヒョウモンや小型のスズメガ類が訪花し、夜間に大型のスズメガ類やヤガ類が主に訪花していた。一方、ヤマユリでは日中にアゲハチョウなどのチョウ類、夜間に大型のスズメガ類やヤガ類が主に訪花していた。ヤマユリに対する訪花昆虫の訪花頻度はサクユリに比べて全体的に低い傾向がみられた。これらの訪花昆虫の中で、その体サイズや採蜜行動を考慮すると、サクユリでは夜行性の大型のスズメガ類が送粉に最も寄与していると考えられた。一方、ヤマユリについては、昼間に訪花する大型のチョウ類と夜間訪花する大型のスズメガ類の両方が送粉に寄与していると考えられた。

　これらのことから、サクユリは夕方および深夜に花香を強く放出し、さらに蜜に含まれる糖量も多くして、夜行性昆虫、特に大型のスズメガ類をより多く誘引していると考えられた。一方、ヤマユリは夜間に昼間よりは強く花香を放出するものの、蜜に含まれる糖量についてはほぼ１日変化させずに、夜間に大型のスズメガ類、昼間にチョウ類を誘引していると考えられた。したがって、伊豆諸島産のより白い花をもったサクユリは、本州産のヤマユリに比べて、夜行性の送粉昆虫により強く依存し、夜間に放出する花香や蜜に含まれる糖量を増加させることによって、それらを誘引するのにより適応していることが本研究によって明らかになった。

Abstract

Lilium auratum var. *platyphyllum*, which is endemic to the Izu Islands, has large white petals and emits a strong floral scent, typical of moth-pollinated plants. Nocturnal moths might be important pollinators within the Izu Islands because swallowtail butterflies are absent or rare there. Therefore, in this study, pollination characteristics, including floral color (petals with or without spots), floral scent intensity, nectar secretion, flowering time, and pollinator assemblages, were investigated for insular var. *platyphyllum* and widespread *Lilium auratum* var. *auratum* on the mainland of Japan to clarify the relationship between floral characteristics and pollinators. Measurement of floral scent intensity using an odor sensor indicated that the intensity increased twice in the evening and at night in var. *platyphyllum*, whereas intensity increased once at night in var. *auratum*. Total sugar weight in nectar, which was calculated by nectar volume and sugar concentration, showed that the flowers of var. *platyphyllum* secreted nectar abundantly both in the evening and at night, whereas the flowers of var. *auratum* secreted almost constant amount of nectar through the day. Flower visitor assemblages and their frequencies studied using digital cameras suggested that crepuscular and nocturnal hawkmoths are important pollinators for var. *platyphyllum* in the Izu Islands, where visitation by the diurnal swallowtail butterfly is low. In contrast, both diurnal swallowtail butterflies and nocturnal large hawkmoths are important and effective pollinators for var. *auratum* on the mainland of Japan. These conclusions were also supported by the exclusion experiments of either diurnal or nocturnal flower visitors using mesh bags. The

rates of seed sets of var. *platyphyllum* were significantly higher from nocturnal pollination than from diurnal pollination. In other words, nocturnal visitors deposited more pollen grains on the stigma than diurnal visitors in var. *platyphyllum*. Thus, this study revealed that pollination characteristics of var. *platyphyllum* show more adaptation for crepuscular and nocturnal hawkmoths, which are relatively abundant in the Izu Islands, than var. *auratum*.

Introduction

The flower is an important organ for reproduction in flowering plants. Approximately 87.5% of angiosperms are pollinated by insects and other animals (Ollerton *et al*. 2011). Flowers often have conspicuously colored petals, a floral scent, and floral morphology to attract their effective pollinators. Red colored flowers attract diurnal pollinators such as swallowtail butterflies (Tanaka 1991), whereas those with white petals and a strong scent attract nocturnal pollinators (Pijl & Dodson 1966; Erhardt 1988; Kato 1999; Yokota & Yahara 2012). Thus, floral diversities are closely linked with pollinators.

Entomophilous flowers of the endemic plant species growing on oceanic islands are often different from those of their relatives on the mainland. Number of pollinator species on oceanic islands is usually lower than that on the mainland (Carlquist 1974; Inoue 1993). The pollination characteristics of island plants were shown to be adapted to the available pollinators on the islands (e.g., Carlquist 1974; Feinsinger *et al*. 1982; Inoue & Amano 1986; Yamada & Maki 2014; Yamada *et al*. 2014) and are thus strongly affected by them.

The Izu Islands constitute one of the oceanic islands in Japan and are located from north to south along the southeastern coast of the Japanese mainland, Honshu. Nine islands in the group are relatively large, namely the Izu-Oshima, Toshima, Niijima, Shikinejima, Kozushima, Miyakejima, Mikurajima, Hachijyojima, and Aogashima islands. The northernmost Izu-Oshima Island and southernmost Aogashima Island are approximately 20 km and 260 km from Honshu, respectively. All the islands were formed by volcanic activity and have never been connected to the mainland of Japan (Karig 1975). Therefore, they are true oceanic islands.

Pollination characteristics of endemic plants in the Izu Islands are different from those of relatives on the mainland. On the islands, large pollinating insects such as bumblebees and swallowtail butterflies are absent or rare (Inoue 1993; Mizusawa *et al*. 2014). Flowers of *Campanula microdonta*, which grows on the islands, are much smaller than those of its relative, *C. punctata*, on the mainland. It was reported that such smaller flowers have resulted from adaptation to smaller pollinators in the Izu Islands (Inoue & Amano 1986). It is also known that several endemic plants in the Izu Islands, such as *Calanthe izu-insularis* and *Weigela coraeensis* var. *fragrans*, emit strong floral scents unlike their mainland relatives. The emittance of strong scents is one of the typical characteristics of nocturnal moth-pollinated plants (Pijl & Dodson 1966; Erhardt 1988; Kato 1999; Yokota & Yahara 2012). Nocturnal moths may be important pollinators in the Izu Islands and flowers of endemic plants in the islands might have adapted to exploit the available pollinators.

In the present study, pollination characteristics of *Lilium auratum* var. *platyphyllum*, which is endemic to the Izu Islands, were investigated on these islands. This variety typically has larger white petals and emits a stronger floral scent than its standard variety, *L. auratum* var. *auratum*, distributed in Honshu. Flowers of var. *auratum* have red or brown spots on white petals, whereas those of var. *platyphyllum* have no spots or faint red spots. Previous studies reported that var. *auratum* also emits a strong floral scent at night, and both diurnal swallowtail butterflies and large nocturnal hawkmoths are important pollinators for the variety (Hayashi 2008; Morinaga *et al*. 2009). In contrast, no detailed investigation on pollination characteristics of var. *platyphyllum* and their pollinators has as yet been conducted. Therefore, I attempted to elucidate the pollination characteristics of var. *platyphyllum* in the present study.

The flower of var. *platyphyllum* has evolved several pollination characteristics typical of moth-pollinated species. The whiter flower of var. *platyphyllum* that emits a stronger floral scent might have adapted to nocturnal hawkmoths because these are the typical characteristics of moth-pollinated plants (Pijl & Dodson 1966; Erhardt 1988; Kato 1999; Yokota & Yahara 2012). As noted above, both diurnal swallowtail butterflies and nocturnal large hawkmoths are effective pollinators for var. *auratum* (Hayashi 2008; Morinaga *et al*. 2009). However, diurnal swallowtail butterflies are reported to be absent or rare in the Izu Islands (Inoue 1993; Mizusawa *et al*. 2014). Therefore, nocturnal hawkmoths might be more effective pollinators for var. *platyphyllum* than for var. *auratum*.

The present study focused on five pollination characteristics: floral color (the petals with or without spots), floral scent intensity, nectar secretion, flowering time and pollinator assemblages. Flower colors are important for attracting pollinators because each flower-visiting animal or insect is known to have a specific color preference. Nocturnal visitors cannot visually search for flowers and use olfactory senses instead. Therefore, flowers mainly pollinated by nocturnal visitors are expected to emit a stronger floral scent only at night (Erhardt 1988; Kato 1999; Yokota & Yahara 2012). Nectar is an important reward to pollinators (Thomson 1988); therefore, plants are known to control nectar secretion so that they can attract their effective pollinators and exclude nectar robbers (Kakutani 1993). It is also well known that flowers pollinated by nocturnal visitors often open in the evening, whereas flowers pollinated by diurnal visitors usually open in the morning (Miyake & Yahara 1999). Thus, floral scent intensity, nectar secretion, flowering time, and pollinator assemblages can be most important pollination characteristics for *Lilium auratum* var. *platyphyllum*. It is for these reasons that I examined these characteristics and compared them to those of var. *auratum* in the present study. I conducted comparisons between individuals of var. *platyphyllum* possessing petals with or without spots because floral color (with or without spots on petals) may also affect pollinators. In addition, diurnal and nocturnal reproductive successes were measured to determine which diurnal or nocturnal pollinator is more important for the plants.

The present thesis tested the following hypothesis: flowers of var. *platyphyllum* are adapted to attract nocturnal pollinators. The present study aimed to answer the following questions: (1) Do the pollinator assemblages differ depending on floral color (with or without spots on petals) in var. *platyphyllum*? (2) When do the floral scent intensities of the two varieties become the strongest in the day? (3) When are their nectars secreted in the two varieties? (4) When do flowers bloom in the two varieties?

Materials and Methods

Plant materials

In the present study, two varieties of *Lilium auratum* (Liliaceae) were used as plant materials.

Lilium auratum var. *platyphyllum* (Fig. 1) is a perennial herb that is endemic to the Izu Islands in Japan. This plant blooms from early July to mid-August and flowers for 3–5 days. An individual plant possesses one or more flowers that bloom acropetally. Flowers are 25–30 cm in diameter and are typically white in color; however, some flowers have red (usually faint) spots on white petals. In the northern islands of the Izu Island group (Izu-Oshima, Toshima, Niijima, Shikinejima and Kozushima islands), individuals with the spotted flowers and those with the non-spotted flowers grow together. In the southern islands (Mikurajima and Aogashima islands), only those with non-spotted flowers grow. The degree of self-incompatibility in this variety has not yet been reported.

Lilium auratum var. *auratum* (Fig. 2) is the standard variety of var. *platyphyllum* and is distributed on the main Island of Japan (Kinki to Tohoku regions on Honshu Island). This variety is also a perennial herb and grows in sunny sites such as along the edges of forests. It blooms from mid-July to early August and flowers for ~5 days. Each plant possesses 1–10 flowers that bloom acropetally. Flowers are 8–20 cm in diameter and possess red or brown spots (darker than those of var. *platyphyllum*) on white petals. This variety was reported to be an outcrosser (Morinaga et al. 2009).

In the present study, two locations for each varieties were selected as study sites (Fig. 3). The investigated populations for var. *platyphyllum* were located in sunny sites along the foot of Mt. Miharayama on the Izu-Oshima Island, and in Okabe, Aogashima Island. The investigated populations for var. *auratum* were located in sunny sites along the forest edge in Hachioji, Tokyo Prefecture, and in Ranzan, Saitama Prefecture, Honshu.

Artificial pollination experiments

To determine the degree of self-incompatibility in each variety of *L. auratum*, artificial pollination experiments were conducted on the Izu-Oshima and Aogashima islands for var. *platyphyllum*, and at Hachioji for var. *auratum*. The following three treatments were performed: outcrossing, self-pollination, and open-pollination. Each treatment was conducted for one flower from different individuals. Pretreatment for the outcrossing and self-pollination treatments included removal of anthers from each flower before dehiscence of anthers and covering stigmas of the flowers with non-woven tape (FC non-woven tape, Hakujuji, Japan). For the outcrossing treatment, 24 flowers of var. *platyphyllum* on Izu-Oshima Island, nine flowers of var. *platyphyllum* on Aogashima Island, 39 flowers of var. *auratum* in Hachioji and eight flowers of var. *auratum* in Ranzan were used. For the self-pollination treatment, 22 flowers of var. *platyphyllum* on Izu-Oshima Island, eight flowers of var. *platyphyllum* on Aogashima Island, and 38 flowers of var. *auratum* in Hachioji were used. For open-pollination, 56 flowers (n = 23, n = 20, n = 13 in 2014, 2015, and 2016, respectively) of var. *platyphyllum* on Izu-Oshima Island, 34 flowers (n = 8 and n = 26 in 2015 and 2016, respectively) of var. *platyphyllum* on Aogashima Island, 14 flowers of var. *auratum* in Hachioji, and

18 flowers of var. *auratum* in Ranzan were used. To estimate the number of seeds that developed in each sample flower, the developed fruits were collected from mid-October to late November and the numbers of seeds and unfertilized ovules in each sample fruit were counted. Fisher's exact test and Mann–Whitney U test were conducted to examine whether the rates of fruit and seed sets, differed among the three treatments.

Measurement of floral scent intensity

A metal-oxide semi-conductor odor sensor (OMX-SRM, Shinyei, Japan) was used to measure floral scent intensities. This odor sensor can record the intensity of any scents as a compound vector of two different sensitivities measured with semi-conductor sensors. For the measurements, each sample flower was enclosed in a polyethylene bag (48 cm × 34 cm) for 7 min and the intensity of floral scent was recorded. Because both of the varieties of *L. auratum* started flowering in the early morning, the floral scent measurements at 4-h intervals were continued from 7:00 on the first flowering day to 15:00 on the 4th day. The measurements for var. *platyphyllum* were conducted from the July 30 to August 2, 2015 on the Izu-Oshima Island, and during July 7–11, 2015 on the Aogashima Island. The measurements for var. *auratum* were conducted during July 11–14, 2016 in Ranzan.

Measurement of nectar volume and sugar concentration

Nectar volume and sugar concentration in each flower of *L. auratum* was measured using capillary glass tubes (EM minicaps, Hirschmann, Germany) and a refractometer (MASTER-α and -2α, Atago, Japan), respectively. Total sugar weight in nectar was then calculated from the concentration and volume. For the measurements, randomly chosen flowers in the bud stage were bagged using mesh bags (20 cm × 12 cm) to prevent pollinator visits. The measurements at 4-h intervals were continued all day from 7:00 on the flowering start day (at 7:00 on the first day, nectar was merely removed without recording its volume and concentration because the volume may include nectar secreted during the bud period) to 15:00 on the 4th day. For var. *platyphyllum*, the measurements were conducted during August 4–7, 2016 on the Izu-Oshima Island, and for var. *auratum* during July 11–14, 2016 in Ranzan.

Camera tracking of flower visitors and timing of flower opening

Flower visitors were recorded using waterproof digital cameras (WG-20 and -30, Pentax, Japan). In total, 12 cameras (four and five cameras for the flowers with spots and without spots on the Izu-Oshima Island, respectively, and three cameras for those on the Aogashima Island) were used for var. *platyphyllum*. For var. *auratum*, 12 cameras (four and eight cameras in Hachioji and Ranzan, respectively) were used. Photographs of the flowers were automatically taken by setting the cameras with automatic shooting intervals of 2 min. Fresh flowers were selected and photos of the same flower continued to be taken for 24 h or more using an electronic flash all day. On each day, 8–14 and 1–7 flowers of var. *platyphyllum* were photographed on the Izu-Oshima Island and on the Aogashima Island, respectively. For var. *auratum*, 6 and 4–11 flowers were photographed in Hachioji and Ranzan, respectively. In the case that a visiting insect was recorded in a series of successively taken pictures, it was recognized as one visit. In addition, timing of flowering was recorded during photographing of flower visitors. A flower was recorded as "open" when all the petals were visible. The flower visitor tracking for var. *platyphyllum* was conducted during August 5–8, 2015 on the Izu-Oshima Island, and during July 7–12, 2015 on the Aogashima Island. Flower visitor tacking was conducted during July 26–28, 2014 in Hachioji and during July 11–15, 2016 in Ranzan.

Measurements of diurnal and nocturnal reproductive success

To measure the contributions of diurnal and nocturnal visitors to the reproductive success of var. *platyphyllum*, visitor exclusion experiments were conducted in 2016 on the Izu-Oshima Island. In the measurement of diurnal reproductive success, only diurnal visitors were allowed to visit the flowers, whereas nocturnal visitors were excluded by bagging the flowers with mesh bags (20 cm × 12 cm) during the nocturnal phase from 15:00 to 6:00. In the measurement of nocturnal reproductive success, diurnal visitors were excluded by the same method from 4:00 to 19:00, and only nocturnal visitors were allowed to visit the flowers. The numbers of individuals investigated for the diurnal and the nocturnal reproductive successes were 13 and 13, respectively. For both the measurements, the same treatments were repeated for 2 days. When the fruits matured, they were collected and their fruit set per treated flowers and seed sets per ovules were examined. A Fisher's exact test and Mann–Whitney U test with Bonferroni adjustment were conducted to examine whether the relative fruit set and/or relative seed set differed among the treatments excluding nocturnal visitors, excluding diurnal visitors, and open-pollination (no exclusion).

Results

Self-incompatibility and pollinator sufficiency

Rates of fruit set and seed set from self-pollination and outcrossing in each population are shown in Fig. 4. The rates of fruit set from self-pollination were significantly lower than those from outcrossing in all the populations examined. The rates of seed set from self-pollination were also lower than those from outcrossing in all the populations examined.

The rates of fruit set and seed set under outcrossing and open-pollination for each population are shown in Fig. 5. In every population, the rate of fruit set from open-pollination was not significantly different from that through outcrossing. On the Izu-Oshima Island, the rates of seed set from open-pollination were significantly lower than those from outcrossing in 2014 and 2015. On the Izu-Oshima Island in 2016 and on the Aogashima Island in 2015, the rates of seed set from open-pollination were not significantly different from those from outcrossing, though the mean value of the former was lower than the latter. In Hachioji and Ranzan, the rates of seed sets from open-pollination were significantly lower than those from outcrossing.

The daily change of floral scent

The daily changes of floral scent are shown in Fig. 6. Floral scent intensities were low in the morning and lowest at 7:00 or 11:00, either in the flowers of var. *platyphyllum* with or without spots on the Izu-Oshima Island, or in those without spots on the Aogashima Island. Their floral scent intensities then increased and showed two peaks at 15:00 and 23:00, or in the early evening and during the beginning of night. Floral scent intensities were highest at 23:00 during the first 2 days. On the other hand, in the Ranzan population of var. *auratum*, floral scent intensity was low during the day and highest at 19:00 at night during the first 2 days. Thus, peak times of floral scent emittance were different between the two varieties of *L. auratum*.

The daily change in nectar secretion

The daily changes in nectar secretion are shown in Table 1 and Fig. 7. When nectar was examined for var. *auratum*, particularly during the latter period of the study, it was almost always rainy; therefore, it was impossible to measure changes in nectar secretion. Nectar secretion began soon after opening of the flowers and lasted for approximately 3 days in var. *platyphyllum*. The nectar volumes in flowers increased during the night throughout their lifetimes. The maximum value of nectar volume was 85.38 μL (at 23:00 of the first day), whereas the minimum volume was 3.16 μL (at 7:00 of the third day). Meanwhile, the nectar volume of var. *auratum* increased slightly during night throughout the lifetime of its flower. The maximum value of nectar volume was 44.69 μL (at 23:00 of the second day), whereas the minimum volume was 6.56 μL (at 15:00 of the second day) for var. *auratum*.

The sugar concentration of the nectar of var. *platyphyllum* increased before midday, was highest at 11:00 of the second day (41.03%), and lowest at 23:00 of the third day (6.27%). Sugar concentration in the nectar of var. *auratum* increased after midday, was highest at 11:00 of the first day (36.30%), and lowest at 7:00 of the third day (13.60%).

In var. *platyphyllum*, total sugar weight in the nectar showed a peak at 15:00, following which it decreased slightly and was the smallest at 11:00. The maximum value of the total sugar weight was 17.30 mg (at 15:00 of the first day), whereas the minimum weight was 0.37 mg (at 7:00 of the fourth day). In var. *auratum*, total sugar weight remained almost unchanged during the lifetime of its flower; the maximum sugar weight was 7.51 mg (at 23:00 of the second day) and the minimum weight was 1.28 mg (at 11:00 of the third day).

Flower visitors and their visiting frequencies

Digital cameras recorded occurrences of insect visits in 147, 186, and 1,060 occasions on flowers of var. *platyphyllum* with spots on the Izu-Oshima Island, those without spots on the Izu-Oshima Island, and those without spots on the Aogashima Island, respectively (Table 2). Insect visits were observed on flowers of var. *auratum* in 44 and 37 occasions in Hachioji and Ranzan, respectively. These flower visitors could roughly be categorized into four groups of insects: 1) Lepidoptera; 2) Coleoptera; 3) Diptera; and 4) Hymenoptera. Among them, Lepidoptera was likely to be the most effective pollinator considering their larger body size and more effective method of collecting nectar compared to the other groups of insects. The hourly visiting frequencies of Lepidoptera on the flowers of var. *platyphyllum* on the Izu-Oshima Island (flowers with and without spots) and on the Aogashima Island, as well as those on the flowers of var. *auratum* in Hachioji and Ranzan, are shown in Fig. 8. Of the visits, 62 (100%) and 80 (98%) occurred at night, and no visits and only two (0%) visits were observed during the day for the flowers with and without spots of var. *platyphyllum* on the Izu-Oshima Island, respectively. On the Aogashima Island, 66 (44%) of visits occurred at night and 85 (56%) occurred during the day. Small hawkmoths were dominant at twilight and large hawkmoths and noctuid moths were dominant at night for var. *platyphyllum* on both islands, whereas small hawkmoths and small butterflies (*Argyreus hyperbius*) were dominant in the day only on the Aogashima Island. Of visits, five (31%) and 19 (53%) occurred at night on the flowers of var. *auratum*, whereas 11 (69%) and 17 (47%) visits occurred during the day in Hachioji and Ranzan, respectively. Large hawkmoths and noctuid moths were dominant at night for var. *auratum*, whereas large swallowtail butterflies and small butterflies were dominant during the day.

Among these insects, hawkmoths (*Agrius convolvuli*, *Deilephila elpenor*, and others) were observed to carry many pollen grains on their abdomens and wings, following which the pollen was deposited on stigmas in both varieties of *L. auratum* (Fig. 9a, b). Swallowtail butterflies (*Papilio machaon* and others) were also observed to carry pollen grains of var. *auratum* on their wings and to deposit the pollen on stigmas (Fig. 9c). According to visual observation and by digital camera recordings, moths (noctuid and geometrid moths) and small butterflies (*Argyreus hyperbius* and skipper butterflies) that visited the flowers only occasionally made contact with both anthers and stigmas of the two varieties.

Flower opening time

Frequencies of flower opening and dehiscing of anthers of the two varieties of *L. auratum* over different times of the day are shown in Fig. 10. Of the recorded flower openings of var. *platyphyllum* by digital cameras, 18 and 6 occasions were observed on the Izu-Oshima Island and on the Aogashima Island, respectively, whereas 24 and 2 occasions of flower opening for var. *auratum* were observed in Hachioji and Ranzan, respectively. Because only a small number of flowers were observed in Ranzan, flower opening times of var. *auratum* in the two populations are shown together in Fig. 10. Most flowers of var. *platyphyllum* growing on the Izu-Oshima Island opened from 7:00 to 9:00, with dehiscing of anthers predominantly occurring from 9:00 to 10:00. Opening of flowers of var. *platyphyllum* growing on Aogashima Island predominantly occurred at approximately 7:00 whereas dehiscing of anthers occurred predominantly from 9:00 to 10:00. Flowers of var. *auratum* growing in Hachioji and Ranzan opened from 4:00 to 7:00, whereas dehiscing of anthers occurred from 7:00 to 8:00. Time required for the stigma to mature could not be examined in the two varieties; however, because their stigmas secreted mucus on the day of flowering, this would indicate that their stigmas mature at least on the same day of the flowering.

Diurnal and nocturnal reproductive success

Rates of fruit set and seed set of diurnally-pollinated, nocturnally-pollinated, and open-pollinated flowers on the Izu-Oshima Island are shown in Fig. 11. There were no significant differences among the rates of fruit set of diurnally-pollinated, nocturnally-pollinated, and open-pollinated flowers, though the fruit set of the diurnally-pollinated flowers appeared to be lower than those of the nocturnally- and open-pollinated flowers. Rates of seed set of diurnally-pollinated flowers were significantly lower than those of nocturnally-pollinated flowers ($p < 0.01$). There were no significant differences between the rates of seed set of diurnally-pollinated flowers and open-pollinated flowers, and between those of nocturnally-pollinated flowers and open-pollinated flowers.

Discussion

Artificial pollination

The rates of fruit set from self-pollination were much lower than those from outcrossing in both var. *platyphyllum* and var. *auratum* (Fig. 4). These results indicated that both varieties of *Lilium auratum* are self-incompatible. Thus, they require pollinators to reproduce through seeds. Meanwhile, the rates of seed set from open-pollination were significantly lower than those from outcrossing for var. *platyphyllum* on the Izu-Oshima Island (2014 and 2015) and for var. *auratum* in the two populations (Fig. 5). These results indicated that the plants of the two varieties in these localities are under pollen limitation. The rates of seed sets for var. *platyphyllum* on the Aogashima Island were not significantly different between open-pollination and outcrossing; however, the former value remained lower than the latter. The lack of significant difference might merely be because of the small sample size on the Aogashima Island. Thus, the available pollinators do not appear to be sufficient for the two varieties in the localities examined in the present study. Therefore, natural selection operates in the two varieties to facilitate the attraction of more effective pollinators, and their pollination characteristics are expected to be adapted for the available pollinators.

The daily change in floral scent

In var. *platyphyllum*, the intensity of floral scent increased twice in the evening and at night, whereas this occurred only once at night in var. *auratum* (Fig. 6). The result for var. *auratum* is consistent with that of a previous study by Morinaga *et* *al*. (2009). However, Morinaga *et al*. (2009) measured floral scent only twice a day and reported that the intensity was higher at night than during the day. In contrast, in the present study, the intensities of floral scent were measured every 4 h for approximately 4 days just after flowering. The results of the present study first showed that the intensity of var. *auratum* became highest at 19:00, following which it decreased. The daily change of floral scent in var. *platyphyllum* was reported for the first time in the present study. Floral scent intensity in var. *platyphyllum* increased from 7:00 or 11:00, showed maximum peaks at 15:00 and 23:00, and then gradually decreased. The daily change of floral scent does not appear to be different between the flowers with spots and those without spots on the Izu-Oshima Island. Thus, the floral scent of var. *platyphyllum* showed both a crepuscularly- and nocturnally-biased emission pattern, whereas var. *auratum* showed only a nocturnally-biased emission pattern. Because a strong floral scent is one of the typical characteristics of moth-pollinated plants (Pijl & Dodson 1966; Erhardt 1988; Kato 1999; Yokota & Yahara 2012), the results of the present study suggest that flowers of var. *platyphyllum* attract both crepuscular and nocturnal moths, whereas those of var. *auratum* attract only nocturnal moths.

The daily change in total sugar weight in nectar

Nectar secretion patterns were different between the two varieties (Fig. 7). Because nectar volume and sugar concentration can easily be affected by humidity of the ambient air (Corbet *et al*. 1979), the daily change in total sugar weight in nectar is discussed in the present study. The total sugar weight of var. *platyphyllum* was highest at 15:00, following which it gradually decreased, and became lowest at 11:00 the following morning. In other words, the flowers of var. *platyphyllum* abundantly secrete nectar in the evening and at night. In contrast, the total sugar weight remained almost unchanged in var. *auratum* throughout the lifetime of its flowers.

Flowers usually attract pollinators by the following two steps (Miyake 2003): 1) flowers notify pollinators of their existence and location by noticeable visual or olfactory cues and; 2) they provide rewards (nectar, pollen, and others) to the pollinators to maintain frequent and recurrent visits. Flowers often show daily patterns of nectar secretion to attract effective pollinators. The results of the present study suggested that flowers of var. *platyphyllum* attract both crepuscular and nocturnal moths as effective pollinators.

Flower visitors and their frequencies

Effective pollinators and visiting frequencies differed between the two varieties of *L. auratum,* as well as between the islands for var. *platyphyllum* (Fig. 8). Flowers of var. *platyphyllum* on the Izu-Oshima Island were visited by large hawkmoths and noctuid moths at night, and by small hawkmoths at twilight. There were no differences in floral visitor fauna and their visiting frequencies between the flowers with spots and those without spots. On the Aogashima Island, flowers (without spots) of var. *platyphyllum* were also visited by large hawkmoths and large noctuid moths at night, as well as by small hawkmoths and small butterflies (*Argyreus hyperbius*) during the day. Among the visitors to the flowers of var. *platyphyllum*, large nocturnal hawkmoths might be the most effective pollinators, considering their large body sizes, effective nectar collecting behaviors, and visiting frequencies. Small crepuscular hawkmoths (*Macroglossum* sp.) might also be effective pollinators considering their effective nectar collecting behaviors and high visiting frequencies.

On the other hand, the flowers of var. *auratum* in both Hachioji and Ranzan were visited by large hawkmoths, noctuid moths, and geometrid moths at night, and by small and large butterflies during the day. Among the visitors to the flowers of var. *auratum*, large nocturnal hawkmoths and large diurnal butterflies (swallowtail butterflies) might be the most effective pollinators. These results for var. *auratum* are consistent with those of previous studies (Hayashi 2008; Morinaga *et al*. 2009). Hayashi (2008) found that the flowers of var. *auratum* are pollinated by swallowtail butterflies during the day and by large hawkmoths at night. Morinaga *et al*. (2009) found that either diurnal or nocturnal visitors were sufficient for its reproductive success.

In a previous study on pollination strategies in *Clerodendrum izuinsulare*, which grows only in the Izu Islands, and in *C. trichotomum*, which is widespread on the main islands of Japan, as well as on some of the Izu Islands, Mizusawa *et al*. (2012) compared pollination characteristics related to the pollinators within Lepidoptera. They conducted a comparative investigation for pollinator assemblage, floral morphology, and flowering phenology between the two closely related species, and concluded based on their observation of flower visitors that the predominant pollinators for *C. izuinsulare* were diurnal hawkmoths, whereas those for *C. trichotomum* were diurnal Japanese black swallowtail butterflies. In other words, they suggested that Japanese black swallowtail butterflies are important pollinators of *C. trichotomum*, whereas diurnal hawkmoths are important as alternative pollinators in the Izu Islands for the endemic species, *C. izuinsulare*. According to their morphology measurements, the lengths of the corolla tubes of *C. izuinsulare* were longer than those of *C. trichotomum*. It is known that long tubes constitute an important adaptation for hawkmoth pollination. Their observation of flowering phenology by counting numbers of flowering trees determined that the flowering season of *C. izuinsulare* occurred later than that of *C. trichotomum*. Mizusawa *et al*. suggested that the difference in the timing of the adult life cycle stage between diurnal hawkmoths and Japanese black swallowtail butterflies might be associated with the difference in flowering phenology between the two plant species. Therefore, they suggested that the pollination characteristics of *C. izuinsulare* were adapted to the diurnal hawkmoths in the Izu Islands, where the visitation frequency of the original pollinator, swallowtail butterflies, is low.

The results obtained for *L. auratum* in the present study lead almost to the same conclusion. Crepuscular and nocturnal hawkmoths were suggested to be important pollinators for var. *platyphyllum* in the Izu Islands, where the visitation of one of the original important pollinators, swallowtail butterflies, is low. In contrast, both diurnal swallowtail butterflies and nocturnal large hawkmoths were confirmed in the present study to be as important and effective pollinators for var. *auratum* on the mainland of Japan. Therefore, var. *platyphyllum,* which grows in the Izu Islands, was more dependent on the crepuscular and nocturnal hawkmoths than var. *auratum*, which is *L. auratum* standard variety distributed widely in Honshu.

Flower opening time

Flowers of both varieties opened during the early morning, following which their anthers dehisced within 2–3 h (Fig. 10). It was reported that the flowers of *Hemerocallis fulva* and its related species *H. citrine* and *H. fulva*, were usually pollinated by diurnal swallowtail butterflies and that they bloomed during the daytime, whereas those of *H. citrina* were pollinated by nocturnal hawkmoths and bloomed during the night (Matsuoka & Hotta 1966; Hasegawa *et al*. 2006). It may appear that flower opening times of the two varieties of *L. auratum* are advantageous for pollination by diurnal insects, although the flowers of var. *platyphyllum* were seldom pollinated by diurnal visitors. This inconsistency might be because *H. fulva* and *H. citrina* bloom only for a half day, whereas the two varieties of *L. auratum* continue to bloom for several days. In other words, flower opening time may not affect the reproductive successes of var. *platyphyllum*, even if it is not completely fit for receiving crepuscular and nocturnal pollinators.

Diurnal and nocturnal reproductive success

The rates of seed sets of var. *platyphyllum* on the Izu-Oshima Island were significantly higher from nocturnal pollination than those from diurnal pollination. There was no significant differences in the rate of seed set between diurnally-pollinated flowers and open-pollinated flowers, although the values of the former were lower than those of the latter (Fig. 11). In other words, nocturnal visitors to var. *platyphyllum* in the Izu-Oshima Island deposited a greater number of pollen grains on the stigma than diurnal visitors. These data indicated that nocturnal visitors are more effective pollinators for var. *platyphyllum* on the Izu-

Oshima Island. In a previous study of var. *auratum*, no significant differences between the rates of seed set among diurnally-pollinated, nocturnally-pollinated, and open-pollinated flowers were evident (Morinaga *et al.* 2009). Morinaga *et al.* (2009) concluded that either diurnal or nocturnal pollinators were sufficient for var. *auratum*. Therefore, the significant difference observed between diurnal and nocturnal reproductive successes in var. *platyphyllum* obtained for the first time in the present study strongly suggest that the most effective pollinators differ between the two varieties. The hypothesis in the present study, namely that var. *platyphyllum* growing in the Izu Islands are more adapted to the nocturnal pollinators than var. *auratum* growing on the mainland of Japan, is supported by the results.

Conclusion

The present study suggested that crepuscular and nocturnal hawkmoths are effective pollinators of *Lilium auratum* var. *platyphyllum* in the Izu Islands, whereas both diurnal swallowtail butterflies and large nocturnal hawkmoths are the important pollinators of var. *auratum* growing on the mainland (Honshu) of Japan. In the present study, no significant differences were evident according to floral color and the presence or absence of spots on the petals between individuals of var. *platyphyllum* with at least a daily change in floral scent intensity and floral visitors. Instead, this study revealed differences in pollination characteristics between the insular var. *platyphyllum* and its widespread congener on the mainland of Japan, var. *auratum*, such as daily change of floral scent intensity, daily nectar secretion pattern, and actual floral visitors. Taking both the results of the previous studies on var. *auratum* and those of the present study into consideration, I concluded that only nocturnal visitors are effective pollinators for var. *platyphyllum*, whereas both diurnal and nocturnal pollinators are effective for var. *auratum*. Flowers of var. *platyphyllum* emit a strong floral scent, both at twilight and at night, and secrete a larger amount of sugar in their nectar to attract crepuscular and nocturnal hawkmoths. In contrast, those of var. *auratum* emit a strong floral scent only during the night and constantly secrete nectar to attract both large nocturnal hawkmoths and diurnal swallowtail butterflies. Thus, the present study suggested that pollination characteristics of var. *platyphyllum* are more adapted to crepuscular and nocturnal hawkmoths, which are relatively abundant in the Izu Islands, than *L. auratum* var. *auratum*.

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Table 1 Daily change in nectar volume (µL), sugar concentration (%), and total sugar weight (mg) in two varieties of *Lilium auratum*. *Hyphen* indicates there were no data.

Var. *platyphyllum* (N = 4)

	1st day					2nd day						3rd day						4th day		
	11:00	15:00	19:00	23:00	3:00	7:00	11:00	15:00	19:00	23:00	3:00	7:00	11:00	15:00	19:00	23:00	3:00	7:00	11:00	15:00
Volume (µl)	7.81	61.82	84.69	85.38	64.36	18.43	4.90	60.11	50.89	60.00	50.79	3.16	6.15	13.02	20.91	22.08	33.23	4.84	0.00	0.00
Concentration (%)	37.73	26.95	18.58	14.00	11.55	22.13	41.03	21.13	15.60	12.93	11.13	17.80	23.95	20.90	12.10	6.27	6.80	7.90	0.00	0.00
Sugar weight (mg)	3.29	17.30	15.66	11.77	7.45	5.42	1.94	12.74	8.13	8.20	6.54	1.56	1.39	2.11	3.76	1.84	2.24	0.37	0.00	0.00

Var. *auratum* (N = 3)

	1st day					2nd day						3rd day						4th day		
	11:00	15:00	19:00	23:00	3:00	7:00	11:00	15:00	19:00	23:00	3:00	7:00	11:00	15:00	19:00	23:00	3:00	7:00	11:00	15:00
Volume (µl)	7.71	12.08	14.54	19.30	22.44	22.10	12.63	6.56	17.08	44.69	39.52	14.40	8.87	21.72	-	-	-	-	0.00	-
Concentration (%)	36.30	24.83	28.50	20.65	18.45	15.65	18.00	29.00	23.20	16.80	14.00	13.60	14.70	20.90	-	-	-	-	0.00	-
Sugar weight (mg)	1.93	3.07	4.22	3.94	4.16	3.42	2.04	1.90	3.96	7.51	5.53	1.96	1.28	4.54	-	-	-	-	0.00	-

Table 2 Insects that visited flowers of *Lilium auratum* var. *platyphyllum* and var. *auratum* at each location. Numbers shown in the table are those of total visitations, whereas numbers in parentheses are those of the visitors that made contact with anthers and/or stigmas. In both varieties, ants, small Coleoptera, and small moths were also observed as flower visitors; however, they did not make contact with anthers or stigmas. Therefore, they were excluded from this table.

Flower visitors			Location					day/night
Order	Family	Species (size)	Oh(with spots)	Oh(without spots)	Ao	Ha	Ra	
Lepidoptera	Papilionidae	*Papilio machaon* (large)		1 (1/0)		3 (3/2)		day
		Papilio memnon (large)					1 (1/0)	day
		Papilio protenor (large)						day
	Nymphalidae	*Argyreus hyperbius* (small)			52 (7/0)			day
		Parantica sita (large)		1 (1/1)				day
	Hesperiidae	*Thoressa varia* (small)				8 (0/0)	16 (0/0)	day
	Macroglossinae	*Macroglossum saga* (small)	1 (0/0)	3 (1/0)	2 (0/0)			day/night
		Macroglossum pyrrhosticta (small)	3 (0/0)	4 (1/0)	20 (4/0)			day/night
		Macroglossum sp. (small)			10 (4/1)			day/night
	Sphingidae	*Agrius convolvuli* (large)	3 (2/1)	2 (1/0)				night
		Deilephila elpenor (large)	1 (0/0)	2 (2/0)		3 (0/0)	6 (3/1)	night
		Theretra japonica (large)		3 (1/0)	2 (1/0)		1 (1/0)	night
		Theretra nessus (large)					1 (0/0)	night
		Psilogramma incretum (large)	3 (2/1)	2 (2/1)	1 (1/0)			night
		Rhagastis sp. (large)			3 (0/0)		1 (0/0)	night
		Sphinx sp. (large)	1 (1/1)					night
		Sphingidae sp. (large)	4 (3/0)	3 (0/0)	3 (2/0)			night
	Geometridae	*Thinopteryx delectans*				1 (0/0)	3 (1/0)	night
	Noctuidae	*Adrapsa notigera*				1 (0/0)		night
		Macdunnoughia purissima					5 (3/0)	night
		Plusiinae sp.	3 (1/0)	1 (0/0)				night
		Thyas juno			6 (0/0)			night
		Thysanoplusia intermixta	21 (5/1)	34 (3/0)	2 (0/0)			night
		Noctuidae sp.	22 (2/0)	26 (2/0)	48 (4/1)		2 (0/1)	night
	-	Unidentified butterfly			1 (0/0)			day
	-	Unidentified moth			1 (1/0)			night
Coleoptera	Scarabaeidae	Scarabaeidae sp.	2 (2/0)		741 (101/9)	3 (1/0)		day/night
Diptera	-	Unidentified flies	15 (0/0)	69 (3/1)	22 (3/0)	22 (0/0)		day/night
Hymenoptera	Halictidae	Unidentified bees	68 (28/0)	35 (23/0)	146 (4/1)	3 (0/0)	1 (0/0)	day

Fig. 1 Flowers of *Lilium auratum* var. *platyphyllum*. (a) Flowers with red spots; (b) Flowers without spots.

Fig. 2 Flowers of *Lilium auratum* var. *auratum*.

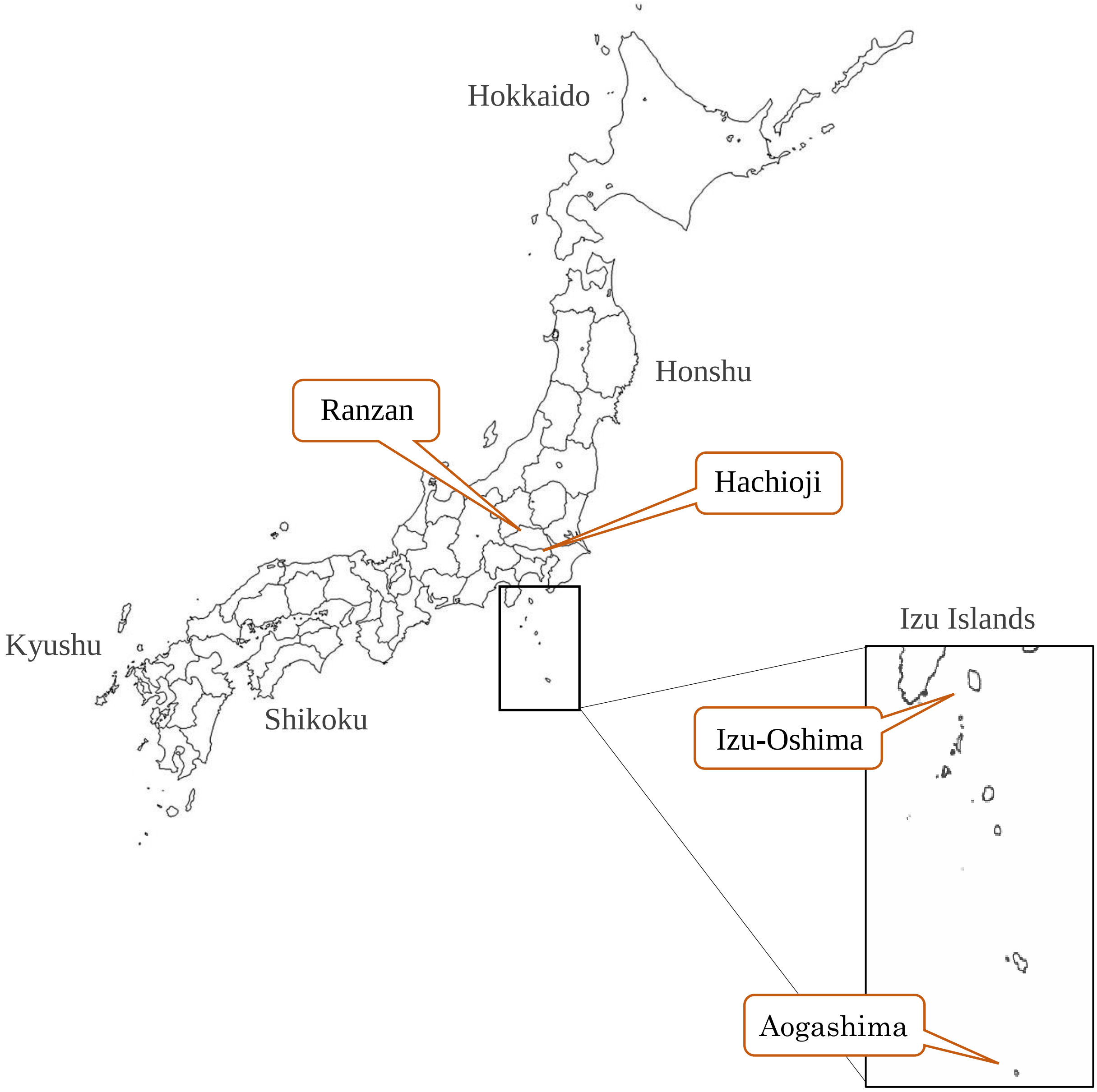


Fig. 3 Four study sites of *Lilium auratum* var. *platyphyllum* and var. *auratum* surveyed in the present study.

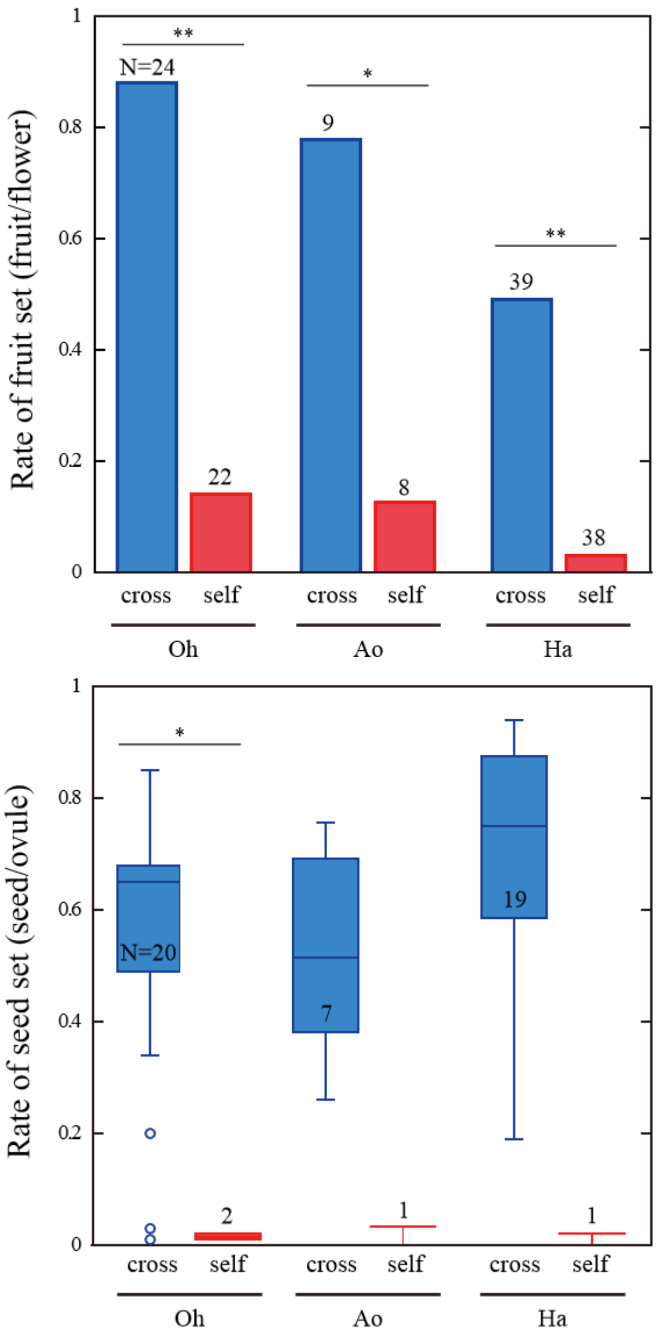


Fig. 4 Rate of fruit sets (top) and seed sets (bottom) subjected to outcrossing (blue bars) and self-pollination (red bars). *Oh* and *Ao* represent *Lilium auratum* var. *platyphyllum* on the Izu-Oshima and Aogashima islands, respectively. *Ha* represents var. *auratum* in Hachioji. The rates significantly differ between outcrossing and self-pollination at $P < 0.05$, marked with an asterisk, and $P < 0.01$, marked with two asterisks (Fisher's exact test for fruit sets and Mann-Whitney U test for seed sets).

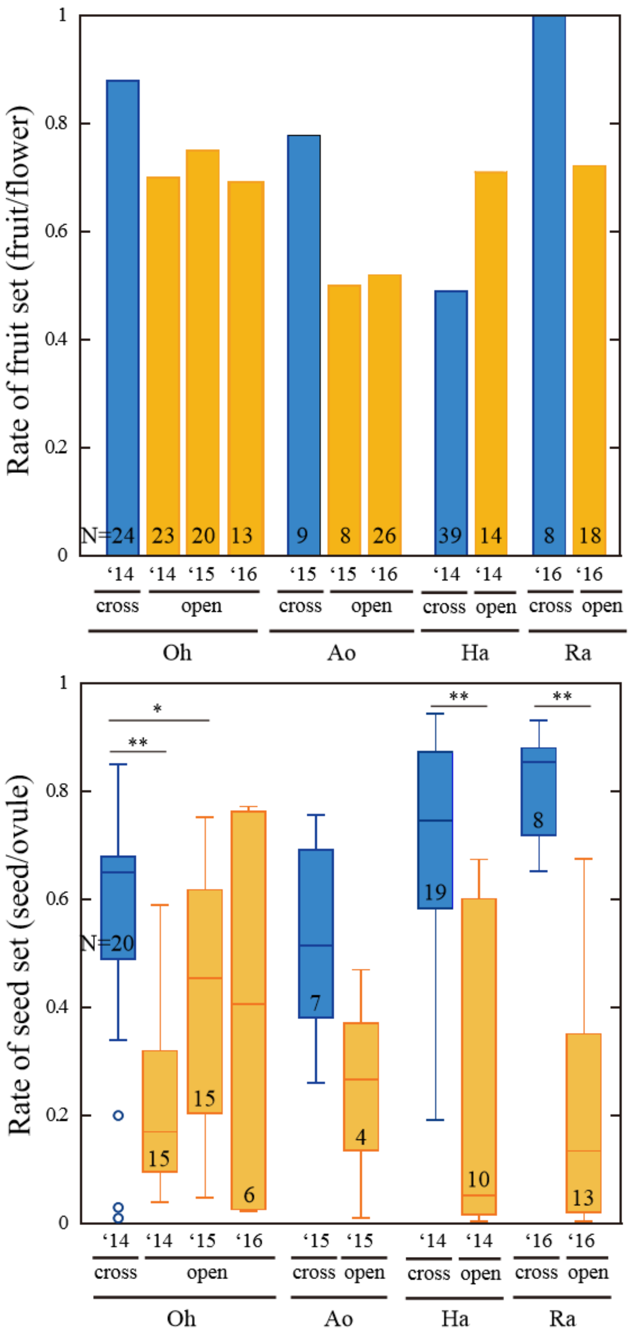


Fig. 5 Rate of fruit sets (top) and seed sets (bottom) subjected to outcrossing (blue bars) and open-pollination (orange bars). *Oh* and *Ao* represent *Lilium auratum* var. *platyphyllum* on the Izu-Oshima and Aogashima islands, respectively. *Ha* and *Ra* represents *Lilium auratum* var. *auratum* in Hachioji and Ranzan, respectively. The rates significantly differ between outcrossing and self-pollination at $P < 0.05$, marked with an asterisk, and $P < 0.01$, marked with two asterisks (Fisher's exact test for fruit sets and Mann–Whitney U test for seed sets).

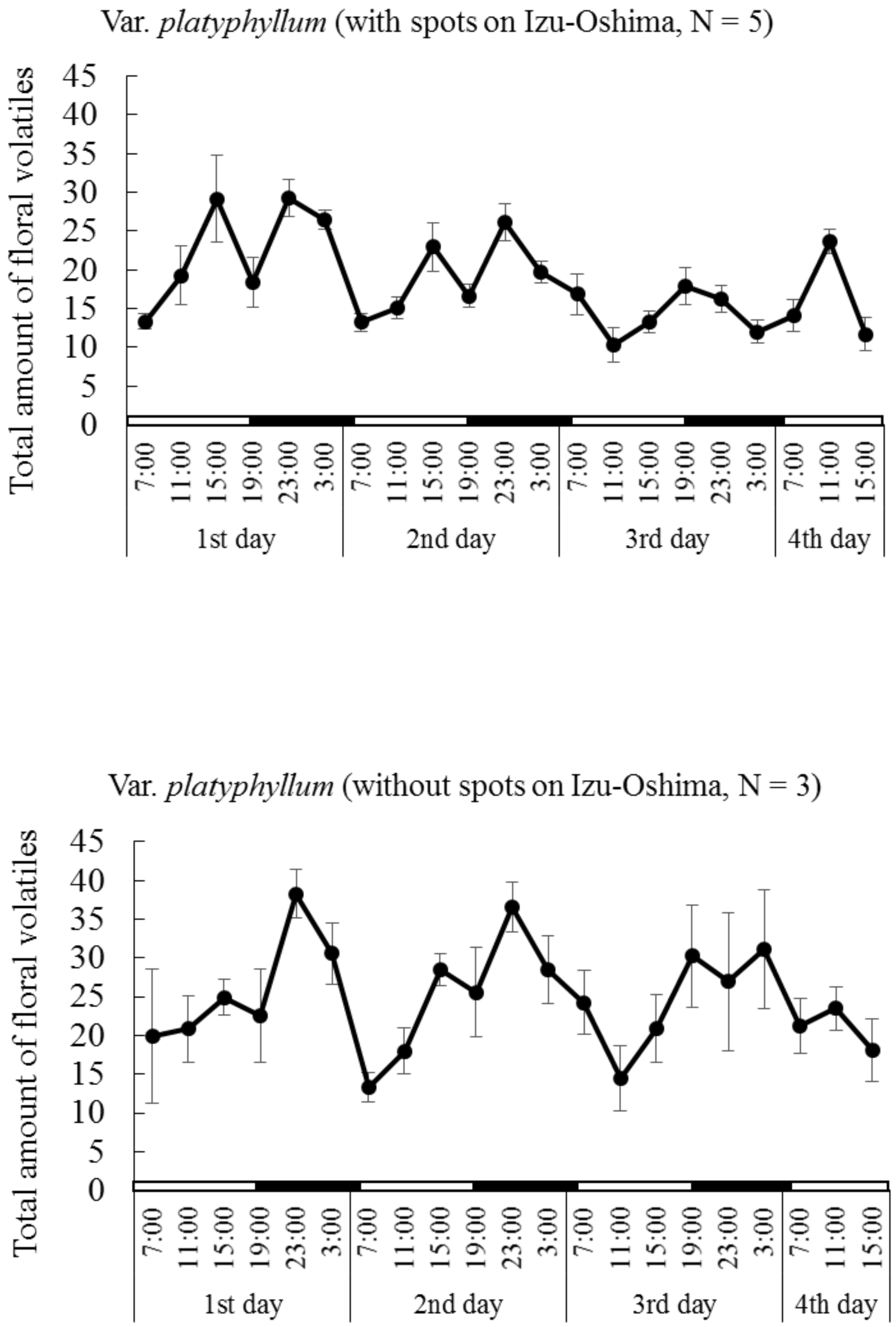
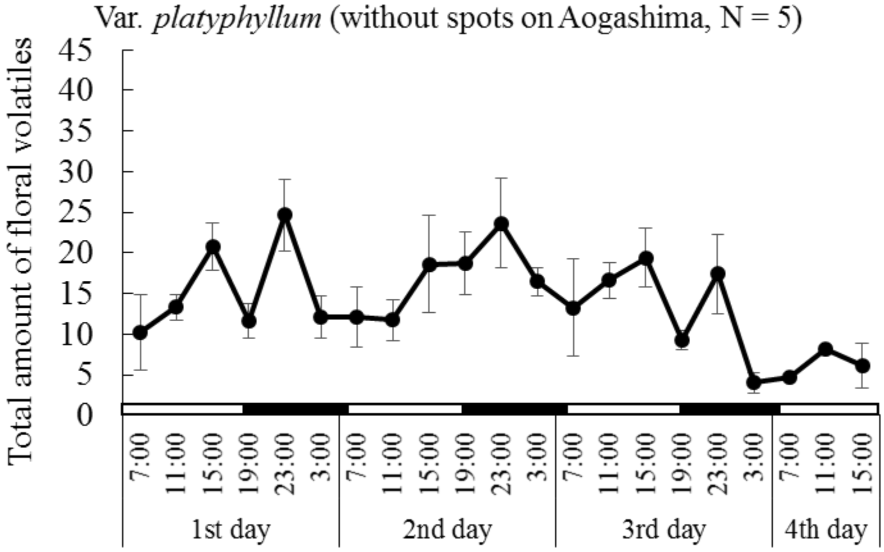


Fig. 6 The daily change of floral scent intensity (mean ± standard error) analyzed by odor sensors. The flowers of *Lilium auratum* var. *platyphyllum* with spots (top) and those without spots (bottom) on the Izu-Oshima Island are shown.



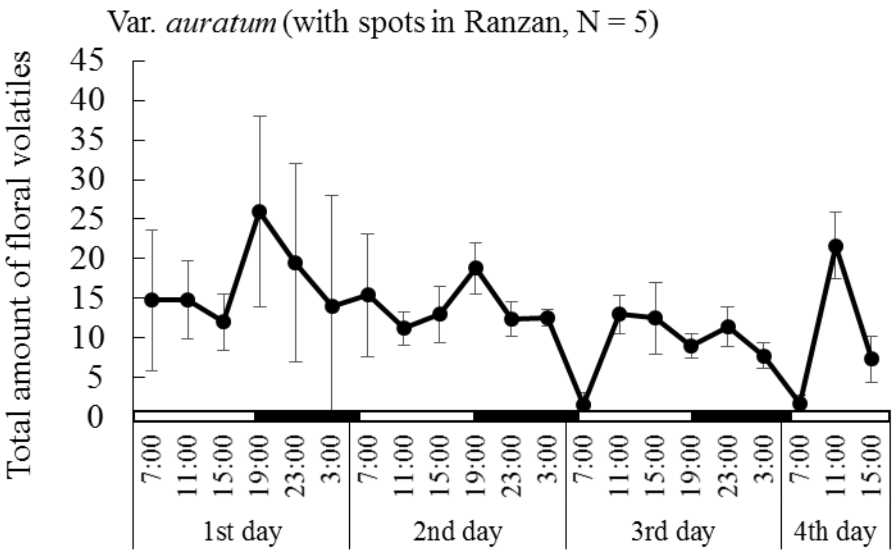


Fig. 6 (Continued) The daily change of floral scent intensity (mean ± standard error) analyzed by odor sensors. The flowers of *Lilium auratum* var. *platyphyllum* on the Aogashima Island (top) and the flowers of *Lilium auratum* var. *auratum* in Ranzan (bottom) are shown.

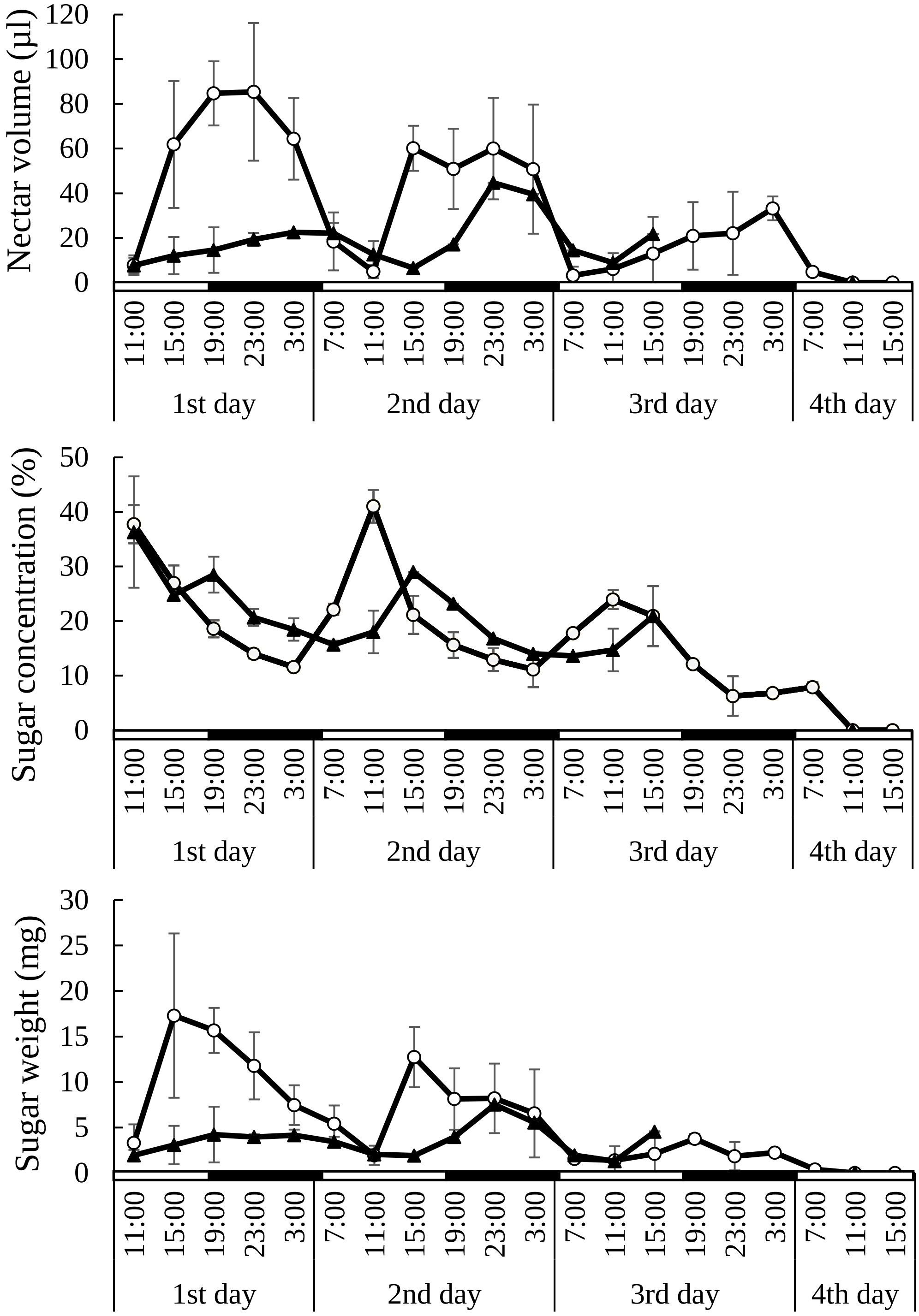


Fig. 7 The daily change of the mean (± standard deviation) nectar volume (top), sugar concentration (middle), and sugar weight calculated from the nectar volume and its sugar concentration (bottom). *White circles* are *Lilium auratum* var. *platyphyllum* on the Izu-Oshima Island and *black triangles* are *Lilium auratum* var. *auratum* in Ranzan. N = 4 and N = 3 for var. *platyphyllum* and var. *auratum*, respectively.

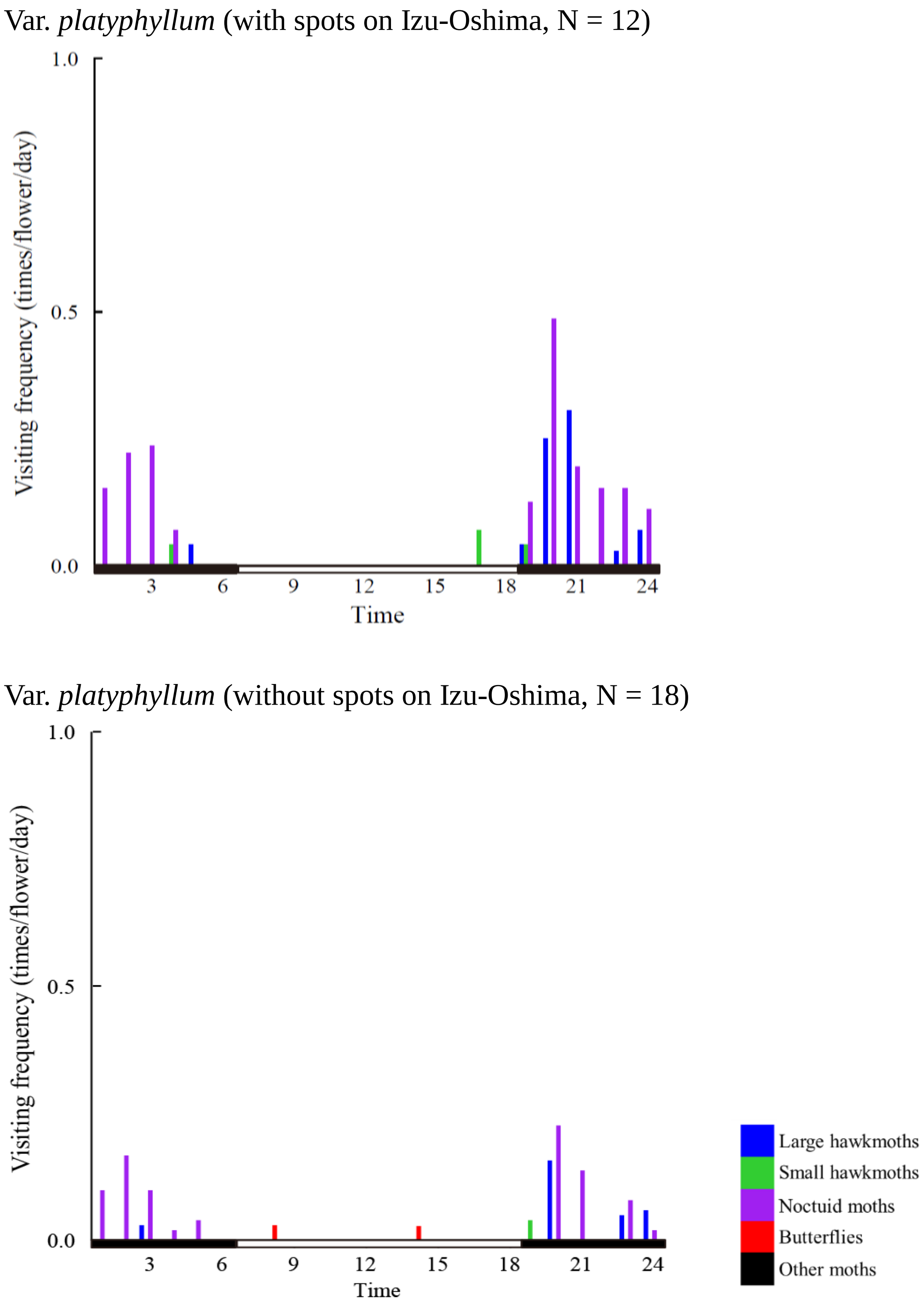


Fig. 8 Hourly flower visitor frequencies of Lepidoptera to the flowers of the two *Lilium* varieties at five study sites. These figures indicate when and what insects visited the flowers of *Lilium auratum* var. *platyphyllum* with (top) and those without spots (bottom) on the Izu-Oshima Island.

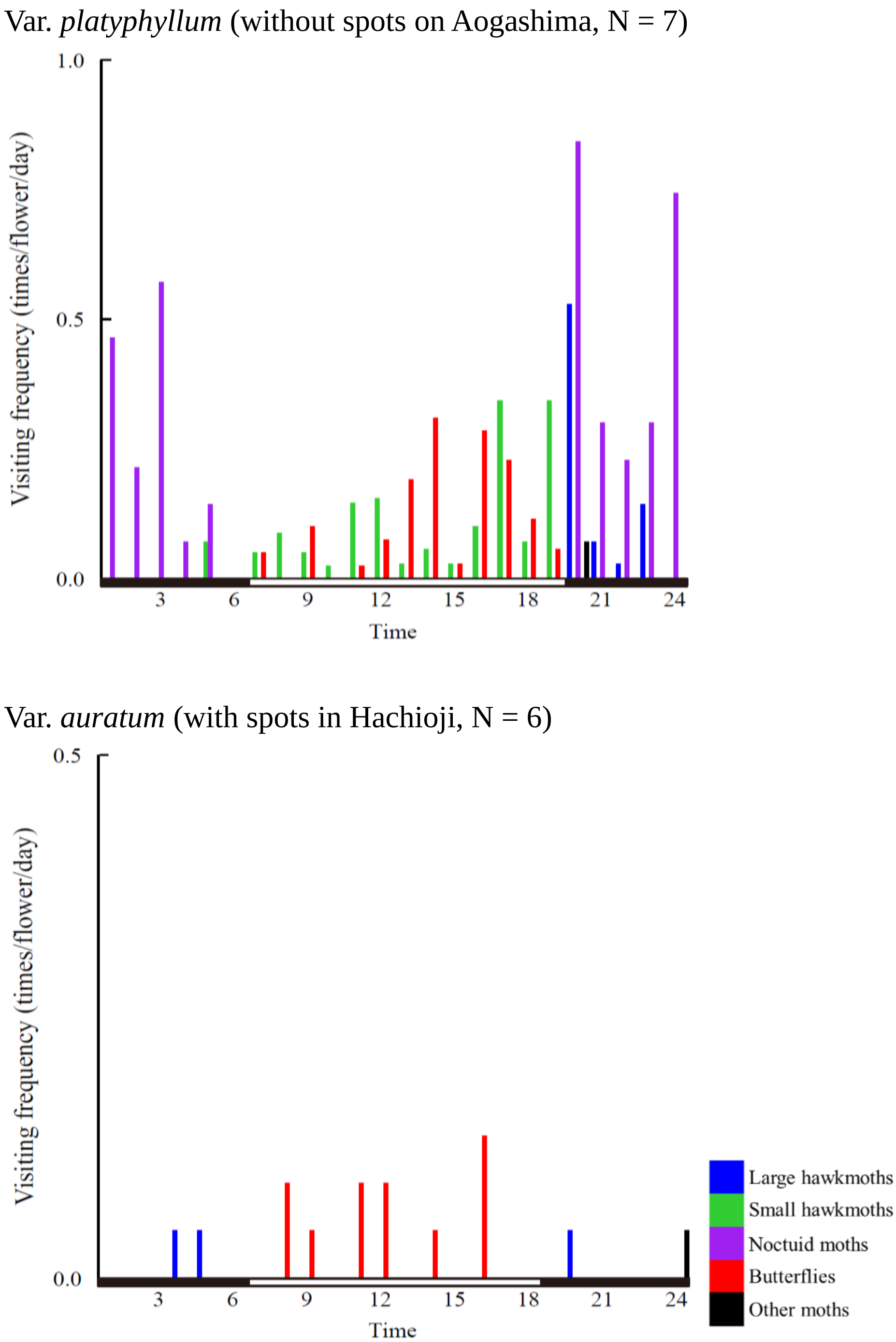


Fig. 8 (Continued) Hourly flower visitor frequencies of Lepidoptera to the flowers of the two *Lilium* varieties at five study sites. These figures indicate when and what insects visited the flowers of *Lilium auratum* var. *platyphyllum* on the Aogashima Island (top) and those of *Lilium auratum* var. *auratum* in Hachioji (bottom).

Var. *auratum* (with spots in Ranzan, N = 13)

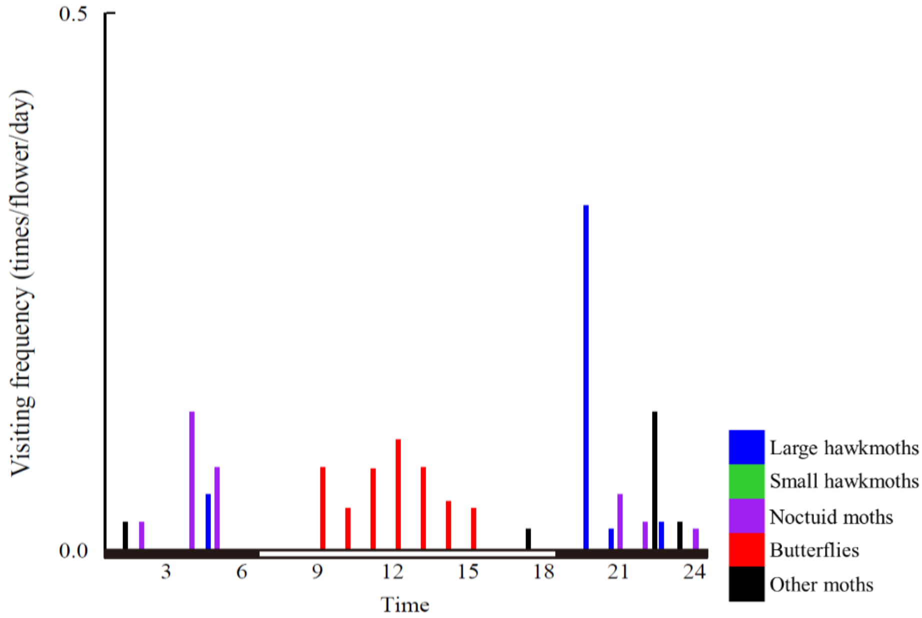


Fig. 8 (Continued) Hourly flower visitor frequencies to the flowers of the two *Lilium* varieties at five study sites. These figures indicate the time and type of insects that visited the flowers of *Lilium auratum* var. *auratum* in Ranzan. *Blue*, *green*, *purple*, *red bars*, and *black bars* represent large hawkmoths, small hawkmoths, noctuid moths, butterflies, and other moths, respectively.

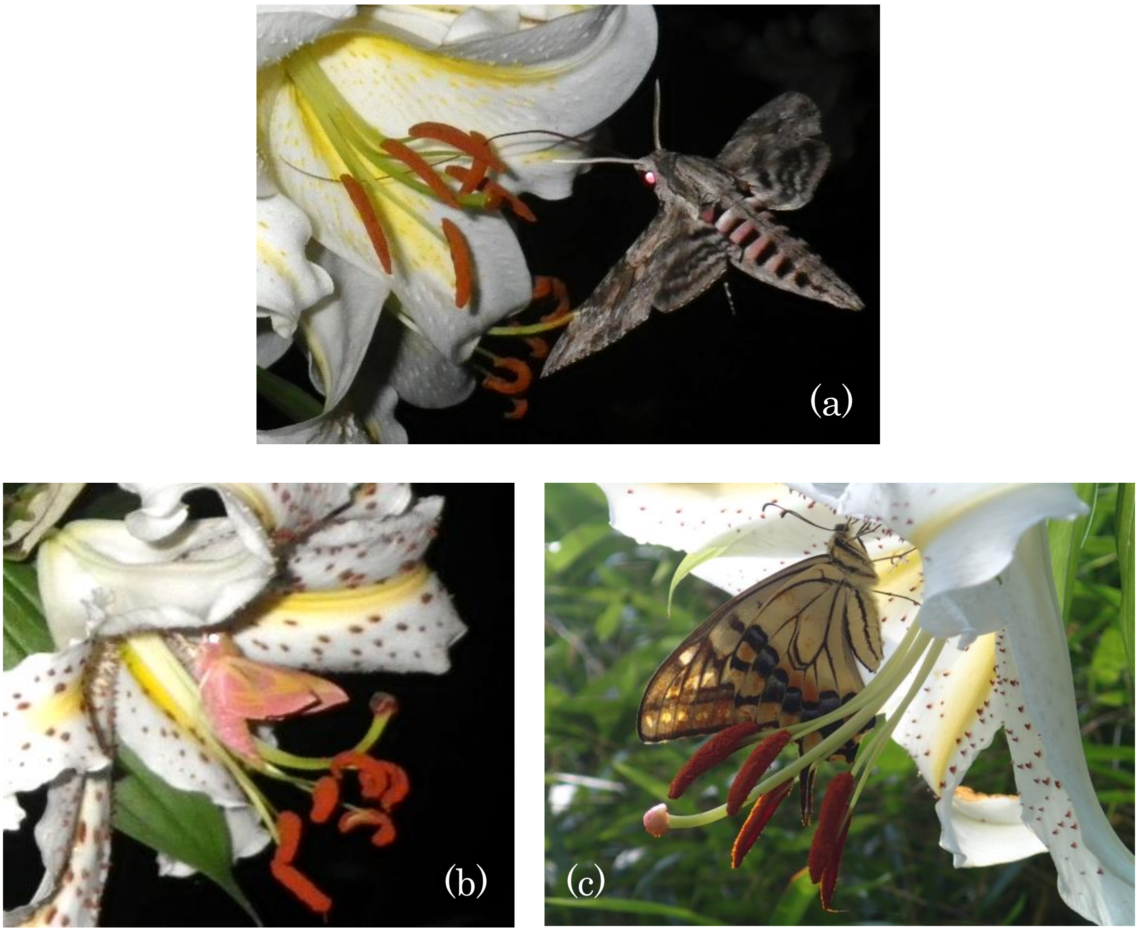
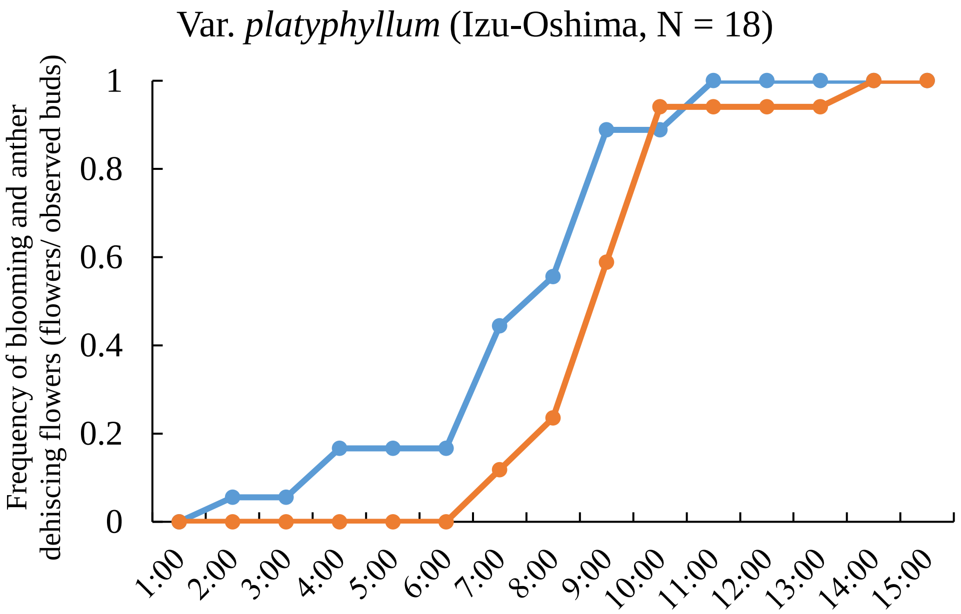


Fig. 9 Flower visitors of *Lilium auratum*. (a) A large hawkmoth (*Agrius convolvuli*) visiting a flower of *Lilium auratum* var. *platyphyllum*; (b) A large hawkmoth (*Deilephila elpenor*) visiting a flower of *Lilium auratum* var. *auratum*; (c) A swallowtail butterfly (*Papilio Machaon*) visiting a flower of var. *auratum*.



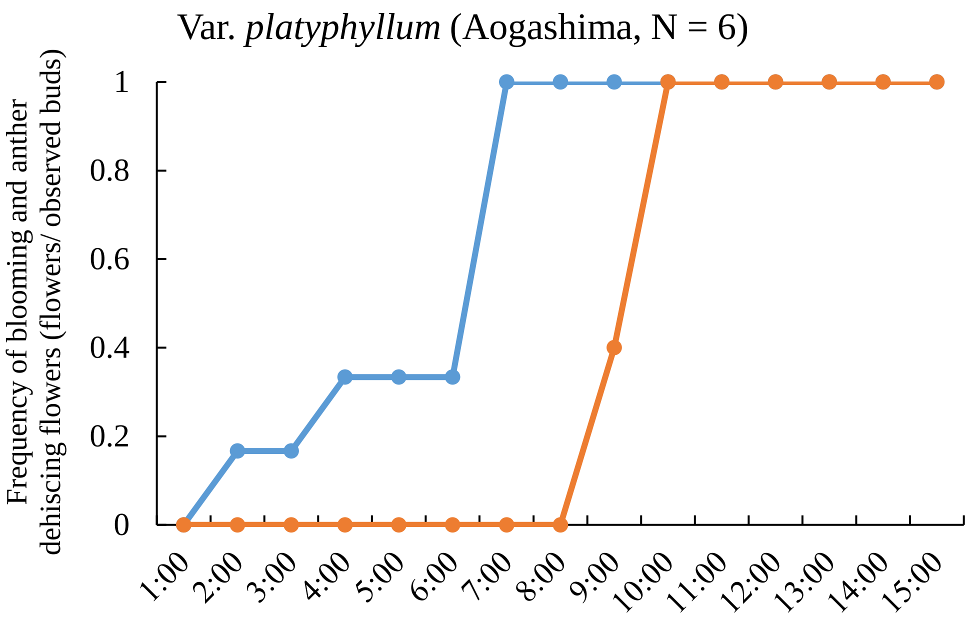


Fig. 10 Flowering frequencies of individuals of the two varieties of *Lilium auratum*, as well as which anthers were dehiscing at each time. The flowers of *Lilium auratum* var. *platyphyllum* on the Izu-Oshima Island (top) and on the Aogashima Island (bottom) are shown.

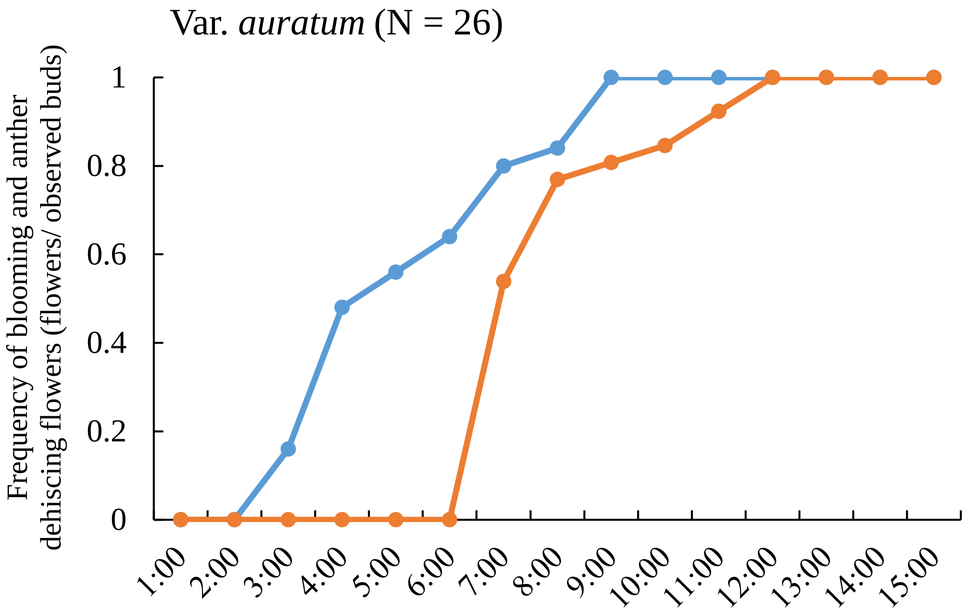


Fig. 10 (Continued) Flowering frequencies of individuals of the two varieties of *Lilium auratum* that underwent flowering and of that of individuals whose anthers were dehiscing at each time. The flowers of *Lilium auratum* var. *auratum* in Hachioji and Ranzan are shown. *Blue lines* represent rates of flowered individuals in each location, and *orange lines* indicate those with dehiscing anthers.

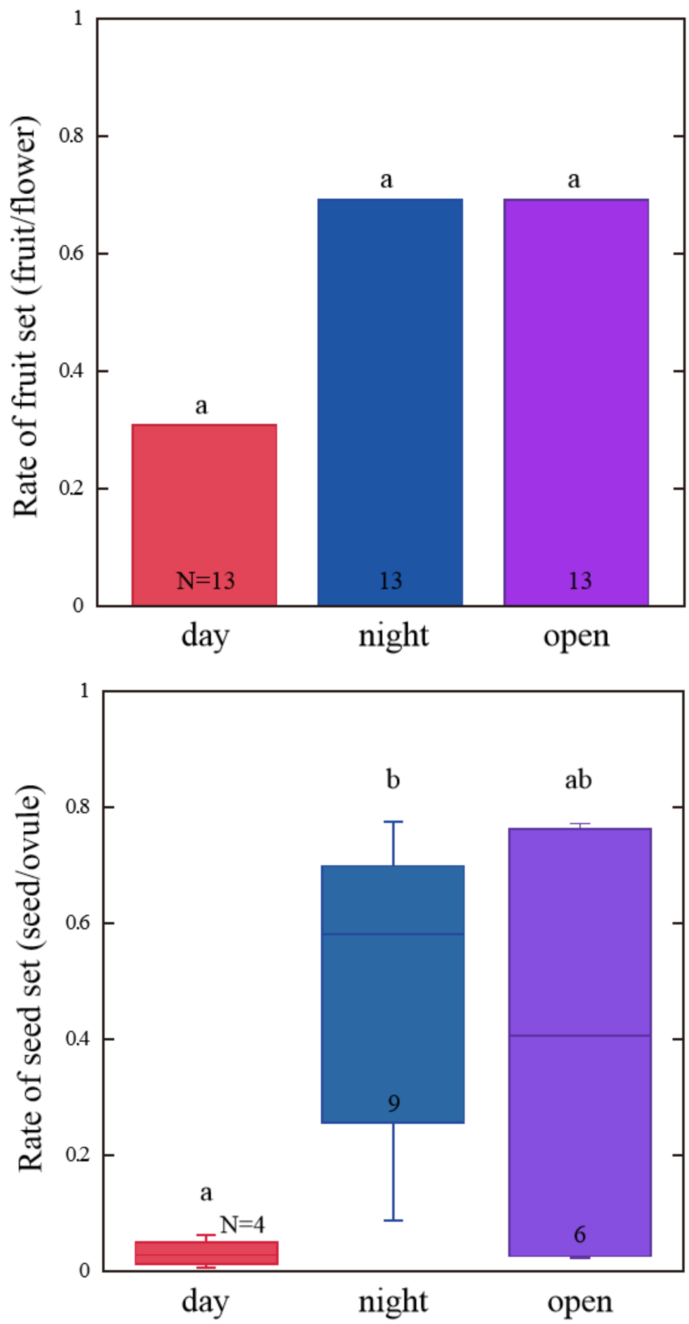


Fig. 11 Rates of fruit set (top) and seed set (bottom) subjected to three different treatments. *Red bars* represent the diurnal pollination (excluded nocturnal pollinators); *blue bars* represent the nocturnal pollination (excluded diurnal pollinators); and *purple bars* represent the open-pollination (no exclusion). The rates with the same letters were not significantly different at $P < 0.01$ by Fisher's exact test for fruit set and Mann—Whitney U test for seed set (with Bonferroni adjustment).